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VITRIFICATION ASSESSMENT OF BUFFALO (*Bubalus bubalis*) OOCYTES: MORPHOLOGICAL AND MOLECULAR ASPECTS

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SUMMARY

Successful cryopreservation of oocytes would preserve the genetic material from superior animals. However, oocyte cryopreservation is relatively less successful comparing to embryos and spermatozoa due to the extreme sensitivity to chilling. Therefore, the objective of this study was to determine the effect of vitrification process before or after oocyte maturation on the expression level of three quality related genes and on the nuclear and cytoplasmic maturation rates of vitrified immature buffalo oocytes. Oocytes were collected and vitrified either immediately after collection as immature oocyte then thawed and matured in vitro (IV group) or vitrified after maturation (MV group). Fresh collected and matured oocytes were used as control group. Maturation rate was determined by cumulus cells expansion (cytoplasmic maturation) and by presence of first polar body (nuclear maturation). The mRNA expression analysis was performed to assess the expression of three quality related genes (SOD1, BAX and MAPK14). The results demonstrated that cryopreservation of immature oocytes did not affect cytoplasmic maturation but significantly reduced the extrusion of the first polar body to 10.6 % compared to 34.1% of control group. BAX transcript showed no significant differences among all groups, although it was down-regulated in IV group and up-regulated in MV ones. However, SOD1 was significantly (P< 0.05) down-regulated in MV group and up-regulated in IV group compared to control group. MAPK14 was significantly (P< 0.05) down-regulated in IV group. In conclusion, this study indicated the deleterious effect of vitrification process on nuclear maturation and expression level of the selected genes in buffalo oocytes.

Keywords: Buffalo; Oocytes; Vitrification; Gene expression; Open-pulled straw

INTRODUCTION

Preservation of gametes and embryos has become an international priority to conserve the livestock genetics and to improve the productivity by applying assisted reproductive techniques. Although significant progress has been made in semen, oocyte and embryo cryopreservation for several animal species, subsequent progress is still limited especially in buffalo. In general, oocyte cryopreservation is relatively poor comparing to embryos and semen due to the complex structure and the extreme sensitivity to chilling (Prentice and Anzar, 2010). Because the volume of a round oocyte (generally, 120 mm in diameter) is larger than that of a spermatozoon, there is a smaller surface-to-volume ratio and a correspondingly higher vulnerability to chilling and intracellular ice formation (Songsasen and Comizzoli, 2009). This resulted in a low percentage of cryopreserved oocytes retaining the ability to undergo normal maturation and fertilization (Le Gal, 1996). In buffalo, due to the high percentage of atretic follicles (Totey *et al*., 1992) and the relatively lower oocyte yield per ovary (Purohit *et al*., 2003); oocyte cryopreservation seems to be the way to preserve and improve the reproductive performance of buffalo. However, buffalo oocytes are supposed to be particularly sensitive to chilling injuries, because of their higher intra-cytoplasmic lipid contents (Boni *et al*., 1992). Therefore, cryopreservation protocols need substantial improvement especially for this species.

Two basic cryopreservation techniques have been widely used; slow freezing, the first to be developed, and vitrification, which, in recent years, has gained a foothold. Unlike the slow freezing method, which requires sophisticated equipment to manage the cooling rate (Saragusty and Arav, 2011), vitrification is a viable and promising alternative that is increasingly becoming more attractive to the commercial sector (Prentice and Anzar, 2010) especially because it can be done cheaply with no need for special equipment. The collected oocytes can be at any level of maturation including oocytes found in primordial, preantral, or antral follicles, each presenting its own special requirements and sensitivities (Carroll *et al*., 1990 and Jewgenow *et al*., 1998).

Recently, different approaches have been applied for analyzing oocyte with different qualities particularly gene expression analysis which hold promise to establish a new tool for selecting competent ones. Therefore, the objective of this study was to determine the effect of vitrification process before or after oocyte maturation on the expression level of three quality related genes and nuclear and

cytoplasmic maturation rates of vitrified immature oocytes in buffalo.

MATERIALS AND METHODS

All chemicals for in vitro cultures and analyses were purchased from Sigma-Aldrich unless otherwise indicated.

Experimental design:

A total of 290 immature buffalo oocytes were collected and vitrified either immediately after collection as immature oocyte (IV) then thawed and matured in vitro (n = 110) or vitrified after maturation (MV) (n = 90). Fresh collected and mature oocytes were used as control group (n = 90).

Collection of ovaries and recovery of oocytes:

Buffalo ovaries were collected from local abattoirs within 2 hrs and transported to the laboratory in physiological saline (0.9% NaCl) containing antibiotic (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin) maintained at 30°C. Ovaries were washed once in 70% ethanol and triple in phosphate buffered saline (PBS). Cumulus oocyte complexes (COC) (n=290) were collected by aspiration of surface follicles present over buffalo ovaries (n=240) using 18-gauge needle attached to a 10-ml syringe. Oocytes received in Hepes-buffered medium 199 ((Medium 199, 22340; Gibco, UK) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Earl's salts, L-glutamine and l-amino acids)) with antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin). Oocytes with uniform cytoplasm and multilayered cumulus cells were selected as good oocytes.

In vitro maturation (IVM) of oocytes:

Oocytes were washed triple in the maturation media consists of Medium199 (31150; Gibco, UK) containing Earl's salts, L-glutamine, supplemented with 10% in activated (56^{o}C /20 min) fetal bovine serum (FBS)(F7524, Sigma, Germany), 0.02 IU/ml FSH (F2293, Sigma, USA), 1µg/ml estradiol 17 β (E2) (E2758, Sigma, USA), 22 µg/ml Na-pyruvate (P-4562; Sigma, USA), 0.3 mM cystine and 50 µg/ml gentamycin. Approximately, 30 oocytes were incubated in 400 µl of maturation media over lied with 400 µl mineral oil in 4 well plates for 24 h at 38.5°C under 5% CO2 atmosphere with high humidity. After 24 h of maturation, oocytes were denuded from cumulus cells by gentle pipetting in maturation media supplemented with 1mg/ml hyaluronidase. Oocyte maturation rate was determined by the cumulus cells expansion and presence of the first polar body according to visual observation (Figure 1).

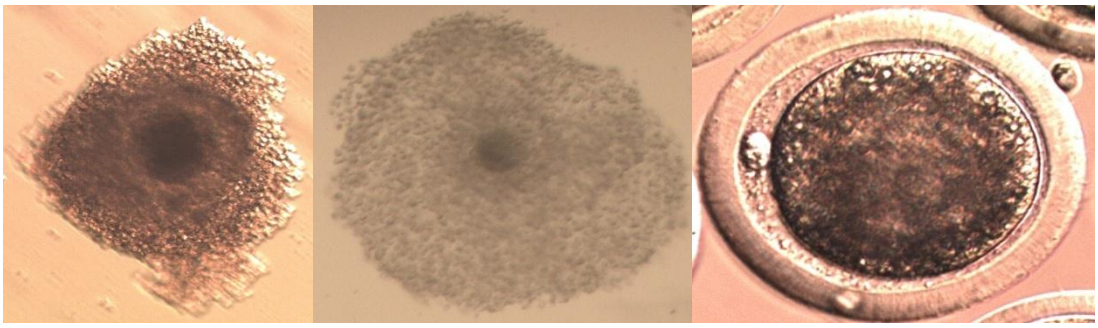

Fig. 1. Illustrated photos of cumulus oocyte complex (coc), mature buffalo oocytes judged by expanded cumulus cells (A) and extrusion of the first polar body (B)

Vitrification of immature and mature oocytes:9

Either mature or immature oocytes were washed twice in holding solution ((HS; Hepes-buffered medium 199 (Medium199, 22340; Gibco, Carlsbad, CA, USA) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Earl's salts, L-glutamine and l-amino acids) + 20% in activated fetal bovine serum) and incubated for 15 min.

Immature and in-vitro matured oocytes were vitrified using the OPS (open-pulled straw) method as previously described by Vieira *et al*. (2002). Groups of four COCs were incubated in the first vitrification solution (VS1; 10% DMSO and 10% EG in HS) for 35 to 40s and then transferred to the second vitrification solution (VS2; 20% DMSO, 20% EG and 0.5 M sucrose in HS) for a further 25s. Instantly, oocytes were loaded onto OPS cryo-devices and plunged into liquid nitrogen (LN2). The time of exposure from VS2 to LN2 was no longer than 30s. OPS were submerged immediately after removing from LN2 into 3 ml pre-warmed (38.5°C) HS plus 1.25 M sucrose (designated T1) and oocytes were smoothly expelled from the OPS.

Oocytes were left in T1 for 1 minute and then, transferred to HS plus 0.625 M sucrose for 30 sec and then transferred to HS plus 0.31 M sucrose for 30 sec. Finally, the oocytes were washed twice in HM for 5 min each and processed for next step (Hadi *et al*., 2011). After thawing, immature oocytes were matured in vitro

and denuded from cumulus cells as described above.

Cumulus free mature oocytes collected from (IV), (MV) and control groups were washed twice in PBS and snap frozen separately in cryo-tubes containing 20 µl of Lysis buffer [0.8 % IGEPAL, 40 U/µl RNasin (Promega Madison WI, USA), 5 m Mdithiothreitol (DTT) (Promega Madison WI, USA)]. Finally, samples were stored at liquid nitrogen at -196°C until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from 3 replicates of each group (30 oocyte / replicate) using PicoPureTM RNA isolation kit (MDS Analytical Technologies GmbH, Ismaning, Germany) according to manufacturer's instructions. For each sample, cDNA synthesis has been performed using oligo (dT) 23 primer, random primer and superscript reverse transcriptase II (Invitrogen, Karlsruhe, Germany). One microlitre of oligo (dT) 23 primer and one microlitre random primer were added to 10µ1 RNA sample and the mixture was incubated for 3 min at 70˚C and then immediately chilled on ice. Eight microlitres of the master mix containing 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT, 1 µl of dNTP (10 pmol/µl) and 0.3 µl of RNase inhibitor and 0.7 µl of Super Script IITM reverse transcriptase (200 unit/µl) was added to the mixture and incubated for 90 min at 42˚C followed by heat inactivation for 15 min at 70˚C. The synthesized cDNA was stored at -20˚C for further use.

Quantitative real-time PCR (qPCR)

Primers were designed based on the cDNA sequences of the four selected transcripts available in GenBank (Table 1) using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). Quantitative analyses of cDNA samples from independent oocytes were performed in comparison with the bovine GAPDH gene (endogenous control), and were run in separate wells using StepOnePlus real-time PCR system (Applied Biosystems). Independent qPCR (3 replicates for each group) was performed in a 20-µl reaction volume containing iTaq SYBR Green master mix with ROX (Bio-Rad Laboratories, Munich, Germany), the cDNA samples and the specific forward and reverse primer. The thermal cycling parameters were set at 95°C for 10 min, 40 cycles of 95°C for 15s. and 60°C for 1 min. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7s interval until the temperature reached 95°C. The comparative cycle threshold (CT) method was used to quantify expression levels as previously described (Bermejo-Alvarez *et al*., 2010).

Table 1. Details of primers used for quantitative real-time PCR

Gene symbol	Gene bank accession Number	Primer sequences	Melting temperature (˚C)	Product size (bp)
SOD1	XM_006053564	F: 5´- AAGGCACCATCCACTTCGAG -3´ R: 5´- TCGTCATTTCCACCTCTGCC -3	60.04	355
BAX	XM_006050928	F: 5´- TGAAGCGCATCGGAGATGAA -3´ R: 5´- CGCTCTCGAAGGAAGTCCAA -3	59.8	253
MAPK14	NM_001102174	F: 5´- TCAGTGGTCTTTCAGCTGCC -3´ R: 5´- TAGGGGGCTACACAACAGGT -3	60.2	270
GAPDH	NM_001034034	F: 5´-ACCCAGAAGACTGTGGATGG-3´ R: 5´-ACGCCTGCTTCACCACCTTC-3´	55	247

Statistical Analysis:

Data were analyzed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., Cary, NC, USA) software package. Maturation rates of oocytes were analyzed by X^2-test. The relative expression data were analyzed using General Linear Model of SAS. Differences in mean values were tested using analysis of variance followed by a multiple pairwise comparison using t-test. Differences of $P \leq 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Effect of cryopreservation on maturation rate

The effect of ethylene glycol and dimethyl sulfoxide in oocyte maturation represented by expansion rate and extrusion of the first polar body is summarized in Table (2). The results of the current study proof that, cryopreservation of immature oocytes, using the above mentioned cryoprotectants, did not affect maturation as indicated by expansion rate compared to control group. However, cryoprotectants reduced extrusion of the first polar body to 10.6 % compared to 34.1% in control group. Our results

showed that, cryopreservation of immature oocytes using ethylene glycol and dimethyl sulfoxide affects maturation rate at nuclear but not at cytoplasmic level. Our results are in agreement with the results obtained by (Mahmoud *et al*., 2010), who found that, proportion of buffalo oocytes reached MII as indicated by nuclear maturation was lower in vitrified group compared to control ones. The negative effect of cryopreservation on nuclear maturation may be attributed to changes occurred at nuclear envelope as mentioned before by (Modina *et al*., 2004). Our results are partially in accordance with the results obtained by (Cooper *et al*., 1998) and (Katie *et al*., 2009) who found that cryopreservation of immature oocytes has no effect on oocyte maturation either at cytoplasmic or nuclear level. The lower rate of extruded polar body in the vitrified group may be explained by the fact that cryopreservation delay the time for oocyte to reach MII as stated before by (Katie *et al*., 2009) and (Guoquan *et al*., 2013). The cryopreservation of mammalian oocytes remains a challenge in most animal species due to its complex structure, where it introduces a series of biophysical and osmotic stresses on the oocytes that can affect several aspects of normal development processes.

Table 2. Maturation rate of cryopreserved immature buffalo oocytes using EG+DMSO compared to control

Groups	Immature oocytes	Mature oocytes (expansion rate) n(%±SE)	Mature oocytes (Polar body rate) n(%±SE)
IV	110	100 (90.9±1.61)	11 (10.6±0.69)[b]
MV	90	80 (88.9±1.11)	28 (35±1.08)[a]
Control	90	82 (91.1±1.11)	28 (34.1±0.79)[a]

Effect of cryopreservation on expression of the selected transcripts

Quantitative PCR (qPCR) was performed to assess the expression of SOD1, BAX and MAPk14 transcripts with respect to vitrification cryoinjury in buffalo oocytes (Figure. 2). After normalization to GAPDH, qPCR analysis indicated that the expression of SOD1 was significantly higher in IV group compared to control group ($P < 0.05$). The expression of MAPK14 was significantly lower ($P < 0.05$) and BAX was lower but the difference was not statistically significant ($P < 0.05$) compared to control group. The effect of vitrification on MV oocytes showed that expression level of SOD1 was significantly lower ($P < 0.05$) and MAPK14 was lower but not significant, while the expression of BAX was higher but not significant in MV group compared to control one. With regard to the expression of BAX in MV oocytes our results were in agreement with these obtained by (Chen *et al*., 2014). It is known that BAX is a pro-apoptotic gene and promotes cell death (Yang and Rajamahendran, 2002). In addition, increased expression of BAX promotes degeneration of oocytes (Rao *et al*., 2012). The trend of this pro-apoptotic gene confirmed the reports which have showed that cryopreservation of immature oocytes was ideal as indicated by the absence of meiotic spindle present as in mature oocytes and the genetic material is confined within the nucleus (Prentice and Anzar, 2010), and this support the theory states that oocytes at various meiotic stages exhibit different sensitivities to cryopreservation. The results are in contrary with that obtained by (Rao *et al*., 2012) who reported that gene expression of BAX was comparable to control levels for in vitro matured oocytes which vitrified-thawed before maturation.

SOD1 is an oxidative stress/damage-related gene and it is reported to be up-regulating upon exposure of embryos to environmental stresses such as vitrification (Castillo-Martín *et al*., 2014) . In the present study, SOD1 was up-regulated in IV group and down-regulated in MV group compared to control group. Up-regulation of SOD1 in IV group is an indication for the stress caused by vitrification process of immature oocytes. Down-regulation of this transcript in oocytes vitrified after maturation (MV group) suggets the harmfull effect of vitrification process that could lead to a degradtions of the stored RNA in the oocyte. It was reported that peroxide levels were negatively correlated with relative SOD1-transcript abundances (Castillo-Martín *et al*., 2014) and (Ho *et al*., 1998) suggested that both the SOD1 and SOD2 enzymes may be the primary targets of superoxide radicals generated during paraquat toxicity. Accordingly, it was noticed that vitrification can induce oxidative stress on oocytes following warming step.

MAPKs are serine/threonine kinases that are fully activated when dually phosphorylated on both tyrosine and threonine residues (Posada and Cooper, 1992). The family of MAPK is known to participate in oocyte maturation in addition to maturation-promoting factor (MPF) (reviewed in Thomas, 1992).

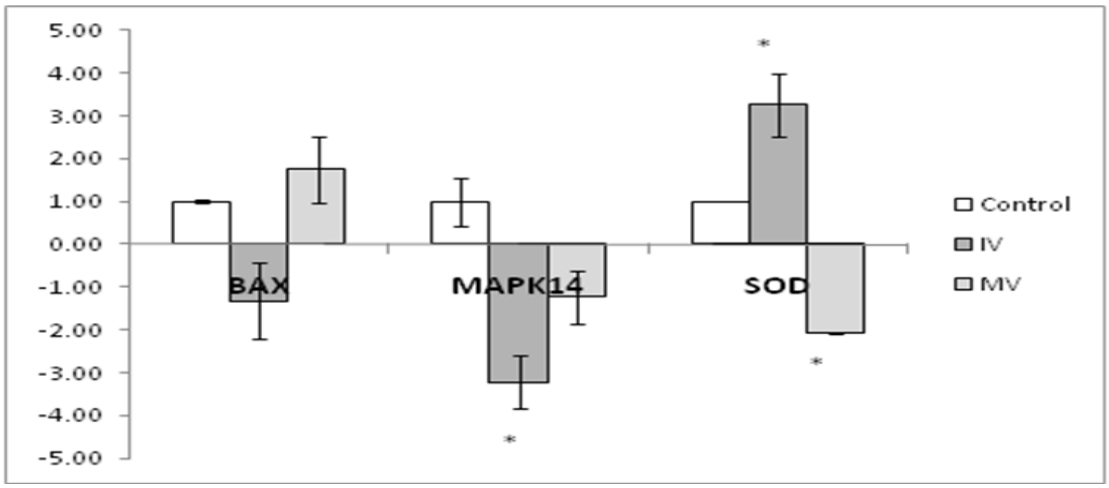

Fig. 2. Relative expression levels of BAX, MAPk14 and SOD1 genes. Values with star symbol show a significant difference compared to control (P < 0.05)

MAPK14 expression which was low in both IV and MV compared to control are in agreement with what stated by (Prentice and Anzar, 2010) in ovine and, who mentioned that poor oocyte cryopreservation has been attributed to the damage to enzymes such as mitogen-activated protein kinase (MAPK), which is critical for oocyte maturation and subsequent embryo development. The same trend was found by (De Blasi, 2011) in bovine who reported that MAPK is down regulated in vitrified oocytes. On the contrary, MAPK activity in sheep cryopreserved oocytes did not differ from that in the control group (Ledda *et al*., 2006).

In fact, all oocytes and embryos suffer considerable morphological and functional damage during cryopreservation, but the extent of the injury as well as differences in post-thaw survival and developmental rates may be highly variable depending on the species, developmental stage and origin. DNA damage in oocyte has been found after cryopreservation and this could be one of the reasons for reduced developmental ability of cryopreserved oocytes (Stachowiak *et al*., 2009 and Men *et al*., 2003a,b). Molecular studies are needed to uncover possible side effects. Regard less of significance of oocyte vitrification the information regarding molecular events occurring subsequent to this process is limited (Habibi *et al*., 2010). Further studies are required to investigate the fertility and developmental ability of MII oocytes following fertilization.

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دراسة تجميد بويضات الجاموس المصري وتاثيره على المستوي الجزيئي والمورفولوجي.

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نجاح عملية حفظ البويضات بالتجميد من شأنه حفظ الاصول الوراثية للحيوانات المميزة. ومع ذلك فإن البويضات تعتبر اقل كفاءة في عملية الحفظ بالتجميد مقارنة بعملية تجميد الاجنة أو الحيوانات المنوية، وذلك نظراً لحساسيتها الشديدة للتجميد. أجريت هذه الدراسة بهدف دراسة تاثير عملية التجميد قبل وبعد انضاج البويضات وذلك على مستوي التعبير الجيني لعدد ثلاثة جينات مرتبطة بجودة البويضات وأيضاً على مستوي معدلات النضج (السيتوبلازمي & النووي) لبويضات الجاموس المصري.

تم تجميع البويضات وتجميدها مباشرة في صورة بويضات غير ناضجة ثم إسالتها وإنضاجها في المعمل (مجموعة IV)، أو تجميدها بعد الإنضاج في المعمل (مجموعة MV)، وذلك مع تجميع مجموعة من البويضات وإنضاجها في المعمل لتستخدم كمجموعة كنترول للدراسة. تم حساب نسبة الإنضاج بواسطة تباعد الخلايا المحيطة بالبويضة (النضج السيتوبلازمي) وأيضاً ظهور الجسم القطبي الأول (النضج النووي). تم إجراء تحليل mRNA لتقييم التعبير الجيني لثلاثة جينات مرتبطة بجودة البويضات وهي (SOD1, BAX and MAPK14). أوضحت النتائج أن تجميد البويضات قبل النضج لم يؤثر على النضج السيتوبلازمي لكنه خفض نسبة ظهور الجسم القطبي الاول بصورة معنوية إلي ١٠,٦% مقارنة بـ ٣٤.١% للمجموعة الكنترول. لم يظهر التعبير الجيني لجين الـ BAX أية فروق معنوية بين كافة المجموعات، بينما أظهر جين الـ SOD1 إنخفاض في التعبير الجيني بصورة معنوية في مجموعة MV وإرتفاع بصورة معنوية في مجموعة IV مقارنة بالمجموعة الكنترول، كما أظهر جين MAPK14 إنخفاض معنوي في التعبير الجيني في مجموعة IV. أكدت هذه الدراسة التأثير الضار لعملية حفظ البويضات بالتجميد على الإنضاج النووي للبويضات وكذلك على مستويات التعبير الجيني لعدد من الجينات في بويضات الجاموس المصري.